X-RAY MICROANALYTICAL MAPPING OF THE INTRACELLULAR DISTRIBUTION OF POLLUTANT METALS

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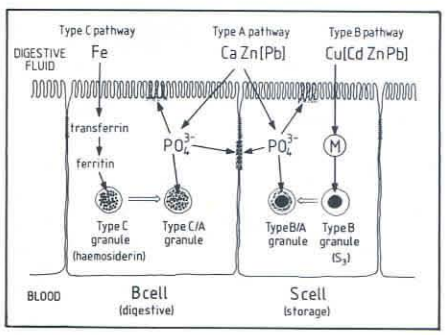

Figure 2. Schematic diagram showing the three pathways of accumulation of metals from the digestive fluids by the hepatopancreas of woodlice.

Figure 1. Dysdera crocata attacking a woodlouse (Porcellio scaber) of 1 cm in length.

Researchers in the Pure & Applied Zoology Department of Reading University have, for several years, been studying the uptake and effects of metals on invertebrate animals which live near to the soil surface. This work has enabled models to be developed for the transport and accumulation of a range of metals at the ecosystem, individual animal and cellular levels[1].

In our research on terrestrial invertebrates, model animals have included centipedes[2] and the common garden snail *Helix aspersa*[3]. However, in this article we shall restrict our discussion to two other common soil animals, woodlice *(Oniscus asellus* and *Porcellio scaber)* and the woodlouse-eating spider *Dysdera crocata* (a photograph of which has also appeared on P.47 of the November 1988 issue of *Microscopy & Analysis*). Fig. 1 shows a specimen of *Dysdera* in the process of attacking a *Porcellio scaber*.

Woodlice have an unrivalled ability to accumulate metals and in contaminated sites, their 'hepatopancreas' (the woodlouse equivalent of a liver) may contain concentrations of zinc, cadmium, lead and copper in excess of 1.2 per cent, 0.4 per cent, 2.5 per cent and 3.4 per cent of the dry weight respectively with no apparent ill effects[4]. *Dysdera* have been maintained in our laboratory on a diet of these contaminated woodlice for several years. Clearly, woodlice and spiders possess sophisticated detoxification mechanisms which enable them to survive concentrations of metals in their body tissues which would kill most other animals.

In this article we describe these mechanisms and show how the technique of x-ray microanalytical mapping has enabled us to develop models for the intracellular detoxification of metals in terrestrial invertebrates.

Preparation of Specimens

Woodlice were collected from a wide range of uncontaminated and metal-polluted sites (principally the region close to a primary zinc, lead and cadmium smelting works at Avonmouth near Bristol), or were fed in a laboratory on diets contaminated with zinc, cadmium, lead or copper. Specimens of these woodlice were fed also to *Dysdera* collected from gardens in Reading. Previous studies have shown that the hepatopancreas of these animals is by far the most important organ of storage of metals so ultrastructural studies have concentrated on this tissue.

Hepatopancreas tissue was fixed in 2.5 per cent glutaraldehyde in 0.1M cacodylate buffer. Specimens for normal transmission electron microscopy (TEM – Figs. 6, 7) were post-fixed in osmium tetroxide and embedded in Spurr's resin. Sections of 60nm in thickness were cut with glass knives on to water with a Reichert Ultracut E Ultra microtome, picked up on uncoated copper grids and stained with uranyl acetate and lead citrate. Tissues for unstained STEM (Figs. 3, 4) or x-ray microanalysis (figs. 8 to 17), were fixed in glutaraldehyde and embedded in Spurr's resin without post-fixation with osmium. Sections of 0.5μm or 1μm were cut on to water, picked up on uncoated aluminium grids and coated with carbon. Further details of preparation techniques have been reported in previous publications[1,4,5].

Analytical Equipment

Sections were examined on a Philips CM12 STEM with EDAX 9900 x-ray analyser at the Philips Electron Optics Centre, Eindhoven. X-ray mapping was performed with a spot diameter of 50nm, a screen resolution of 256×200 or 512×400 pixels and a dwell-time on each pixel of 50 or 100ms. Images (figs. 8 to 17) were photographed directly from the colour monitor with an Olympus OM2SP camera with 100mm lens using Ektachrome 100 35mm transparency film.

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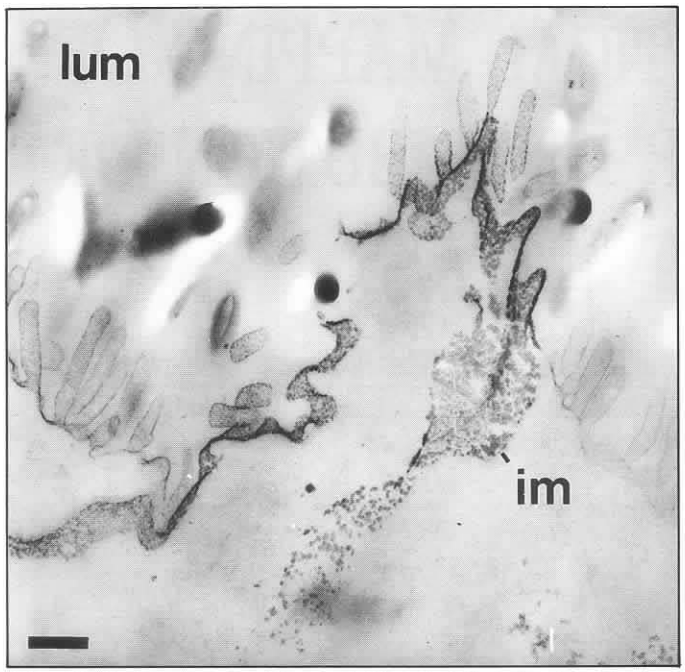


Figure 3. Unstained section (1μm in thickness) of the microvillus border of cells in the woodlouse hepatopancreas. Type A material containing calcium, phosphorus, zinc and lead is present on the membranes. lum, lumen; im, intercellular membranes. Scale bar 0.25μm.

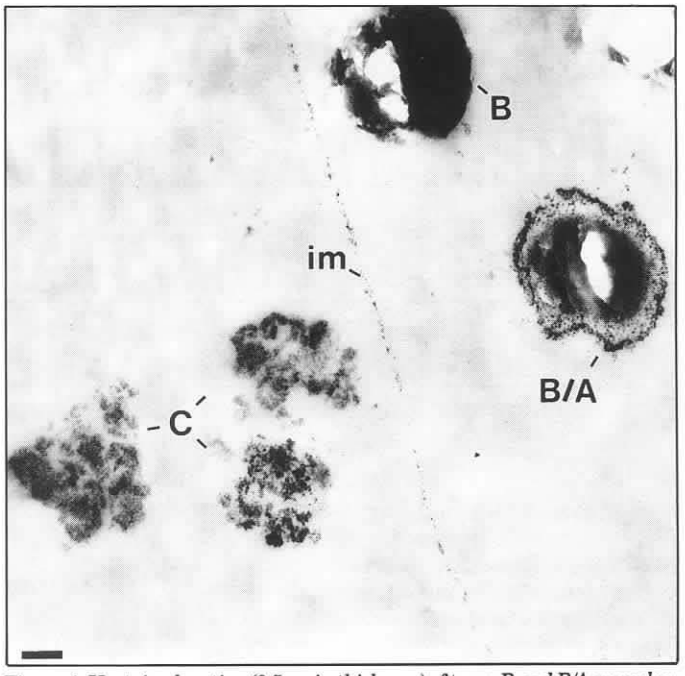


Figure 4. Unstained section (0.5μm in thickness) of types B and B/A granules in an S cell and type C granules in a B cell of the woodlouse hepatopancreas. Deposits of type A material are present on the intercellular membranes (im). Scale bar 0.3μm.

Results

Most biologists working on the effects of metal pollutants on animals classify metals according to the scheme of Nieboer and Richardson[6]. These authors suggested adoption of the classification system originated by chemists, which uses Lewis acid properties (i.e. 'hardness' or 'softness' as acids and bases), to separate metals into class A, class B and intermediate or borderline metals. Under this system, class A metals such as calcium are predominantly oxygen-seeking and class B metals such as cadmium and copper bind preferentially to nitrogen or sulphur-bearing ligands. Borderline metals such as zinc and lead exhibit characteristics of both class A and class B metals. This classification system has proved to be extremely useful in helping us to interpret the intracellular distribution of metals in invertebrates.

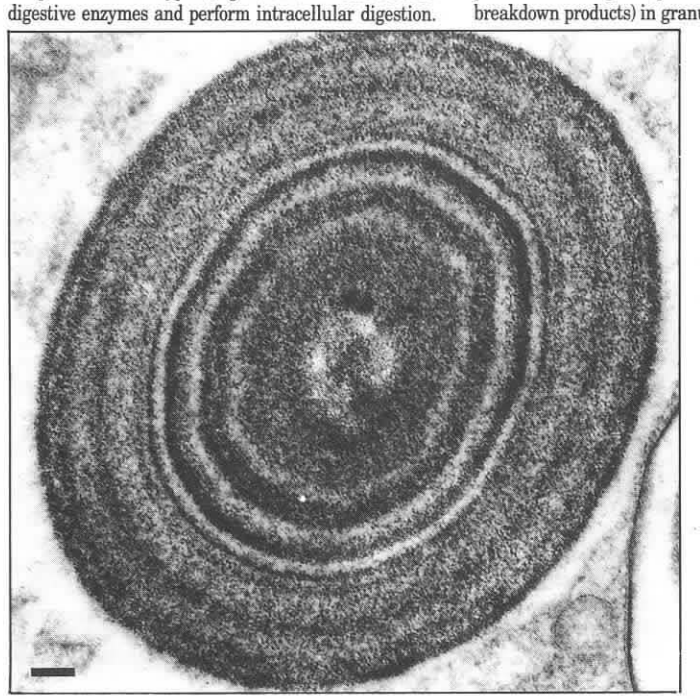
Woodlice

The hepatopancreas of woodlice consists of four blind-ending tubules which open into the foregut[5]. The organ comprises two cell types (Fig. 2). The 'B' cells secrete digestive enzymes and perform intracellular digestion. Large numbers of B cells are broken down and replaced during each 24 hour digestive cycle. The 'S' cells, which have an extremely long residence time in the hepatopancreas, function mainly as sites of permanent storage of metals. Three pathways of metal accumulation by these cells from the digestive fluid in the lumen of the hepatopancreas can be recognised (Fig. 2).

The class A metal calcium follows the *Type A pathway* and is precipitated with phospate to form amorphous deposits of calcium phosphate *(Type A material)* on the cytoplasmic side of the microvilli (Figs. 3, 15) and the intercellular membranes and in the vacuoles containing existing type B granules in the S cells or existing type C granules in the B cells (Fig. 4). The borderline metals zinc and lead may follow these pathways also (Figs. 10 to 15).

The class B metal copper follows the *type B pathway*, probably *via* metallothionein (a cysteine-rich protein with a low molecular weight of about 10^4 daltons) and is deposited with sulphur (probably derived from cysteine breakdown products) in granules with a spherical (Figs. 10 to 14) or hetereogenous structure (Figs. 8, 9). These *Type B granules*, which are found only in the S cells, may have their origin in the lysosomal system as they are often rich in acid phosphatase. The class B metal cadmium and the borderline metals zinc and lead may be detected in these granules also (Figs. 8, 9). The *Type B/A granules* (fig. 2) in the S cells are almost certainly formed from the deposition of type A material around 'mature' type B granules which have ceased to 'grow'. The type B core/type A periphery arrangement has been consisent in every B/A granule we have examined (e.g. Figs. 10 to 14).

Iron follows the separate *Type C pathway* into the B cells only, *via* transferrin (the iron carrier protein) and ferritin (the iron storage protein). Unwanted iron is deposited in vacuoles (probably as haemosiderin) in *Type C granules* (Figs. 2, 4, 8, 9). The *Type C/A granules* (Fig. 2) in the B cells are probably formed by the deposition of type A material into existing type C granules (Figs. 8, 9). Consequently in contaminated woodlice, zinc and lead occur in this material.



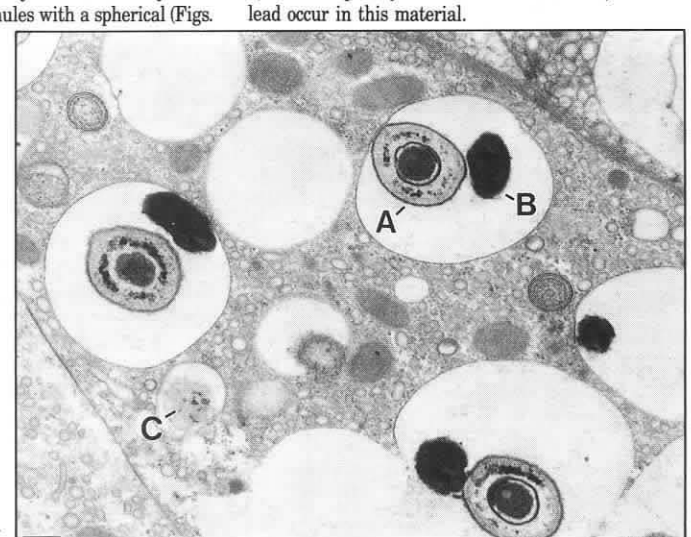


Figure 6. A typical type A granule in a digestive cell of Dysdera crocata. Scale bar 0.12μm.

Figure 7. A digestive cell of Dysdera crocata breaking down at the end of a digestive cycle. The A, B and C granules are eventually deposited in a large excretory vacuole which is voided into the lumen. Scale bar 0.8μm.

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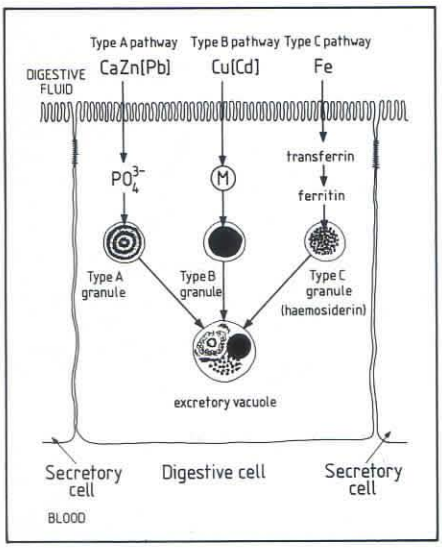

Figure 5. Schematic diagram showing the three pathways of accumulation of metals from the digestive fluids by the digetive cells in the hepatopancreas of Dysdera crocata.

The only woodlice in which we have observed changes to the pathways outlined here are very heavily contaminated individuals when metals which would normally follow one pathway have 'spilled over' into another, due possibly to saturation of uptake sites on the cell membrane (e.g. traces of cadmium in a type C granule – Fig. 9).

Spiders

The hepatopancreas of *Dysdera crocata* consists of many hundreds of blind-ending tubules which open into the gut. The organ comprises two cell types (Fig. 5). The *Digestive* cells perform intracellular digestion and store metals. Large numbers of digestive cells are broken down and replaced during each 24 hour digestive cycle. The *Secretory* cells, which have a much longer residence time in the hepatopancreas, are rich in rough endoplasmic reticulum and are responsible for the secretion of digestive enzymes. These cells are not heavily involved in metal storage. The same three pathways of metal accumulation by the digestive cells can be recognised (Fig. 5).

The class A metal calcium follows the type A pathway and is precipitated with phosphate to form spherical Type A granules which have a characteristic arrangement of concentric layers in thin section (Fig. 6). These granules invariably contain zinc (Fig. 16) and in spiders fed on lead-contaminated woodlice, lead may be detected also.

The class B metal copper follows the type B pathway and forms granules with a similar appearance and composition as the type B granules of woodlice. In spiders fed on cadmium-contaminated woodlice, cadmium may be detected in the type B granules.

Iron is not detected in type A (Fig. 17) or B granules but is present initially in the membrane-bound heterogeneous deposits in the cytoplasm of the digestive cells (Fig. 7). Towards the end of the digestive cycle, the three types of granules can be found inside the same excretory vacuole (Figs. 5, 7) prior to being discharged as waste material into the lumen of the hepatopancreas when the

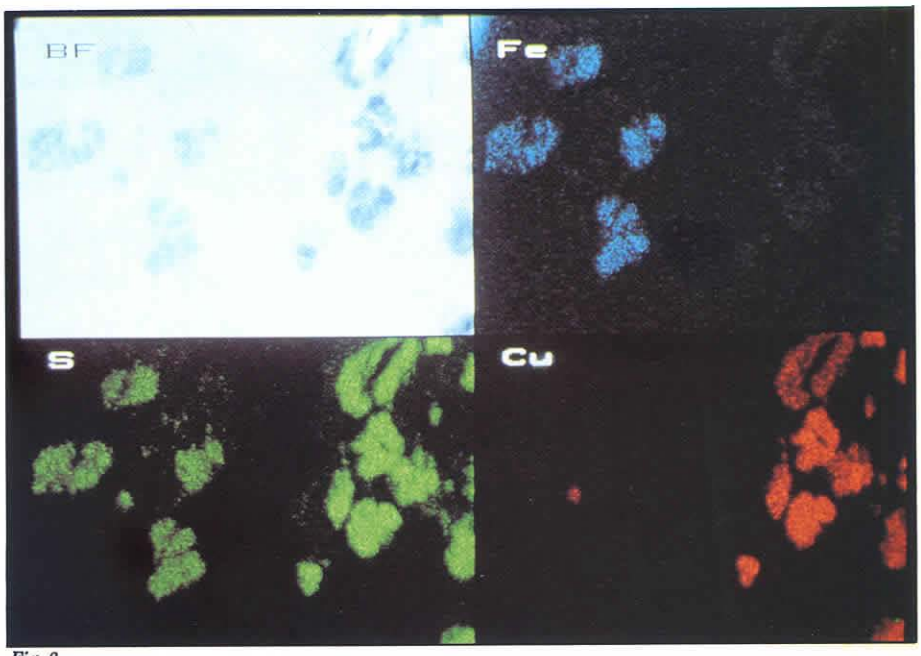

Fig. 8

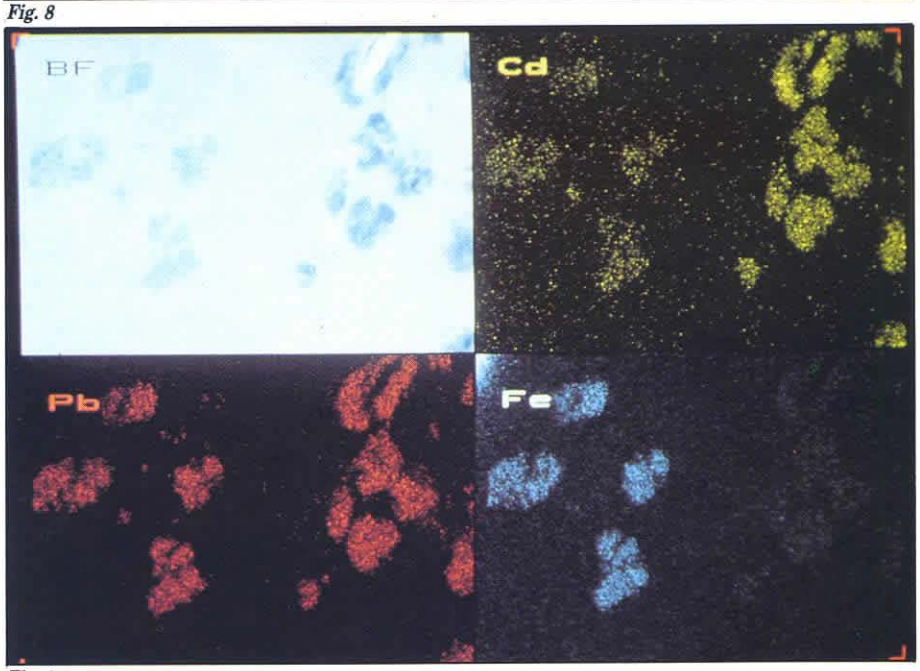

Fig. 9

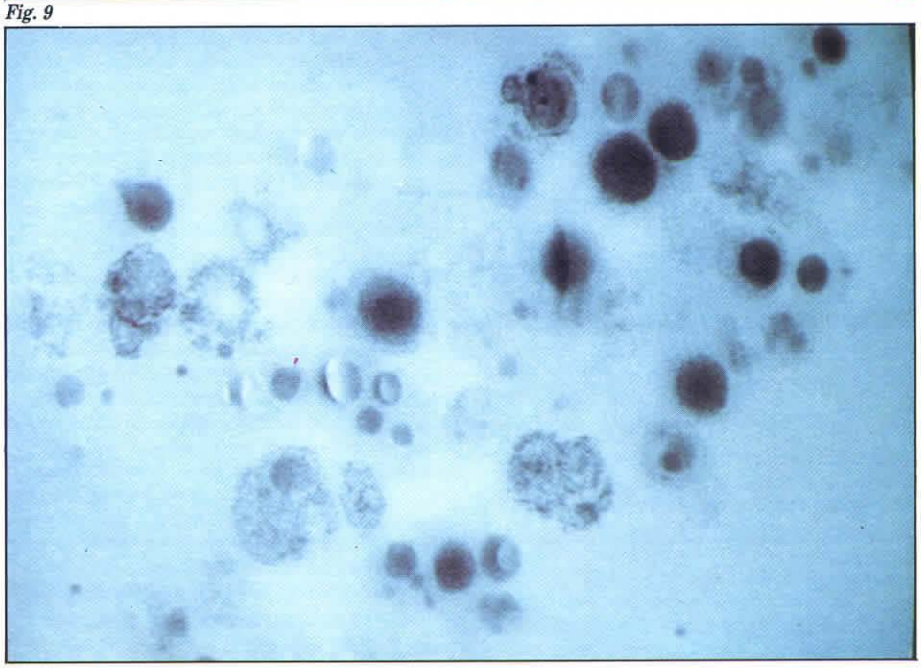

Fig. 10

Figure 8. Bright field STEM image and x-ray maps for iron (Fe), sulphur/lead (S) and copper (Cu) in type B (right) and C granules (left) similar to those shown in Figure 4. HFW of each map 5μm.

Figure 9. As figure 8 but for cadmium (Cd), lead (Pb) and iron (Fe). The woodlouse was collected from close to the Avonmouth zinc, lead and cadmium smelting works.

Figure 10. Bright field STEM image of granules in an S cell of a woodlouse collected from the University Campus. HFW 20μm.

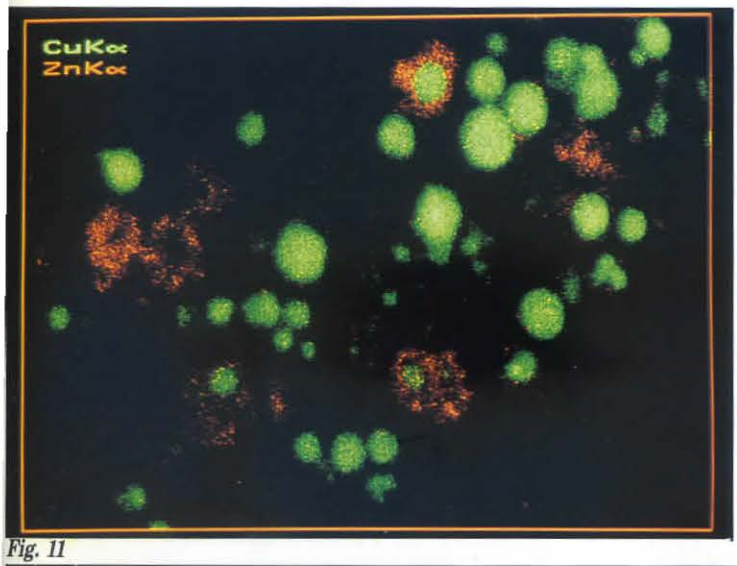

Fig. 11

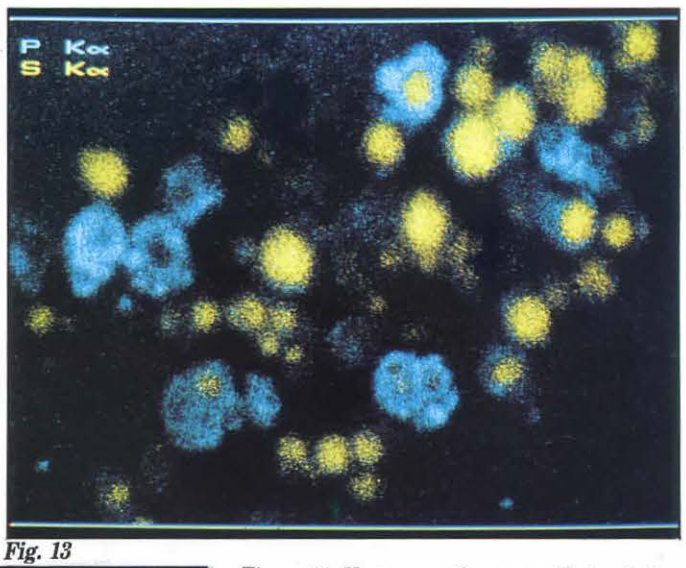

Fig. 13

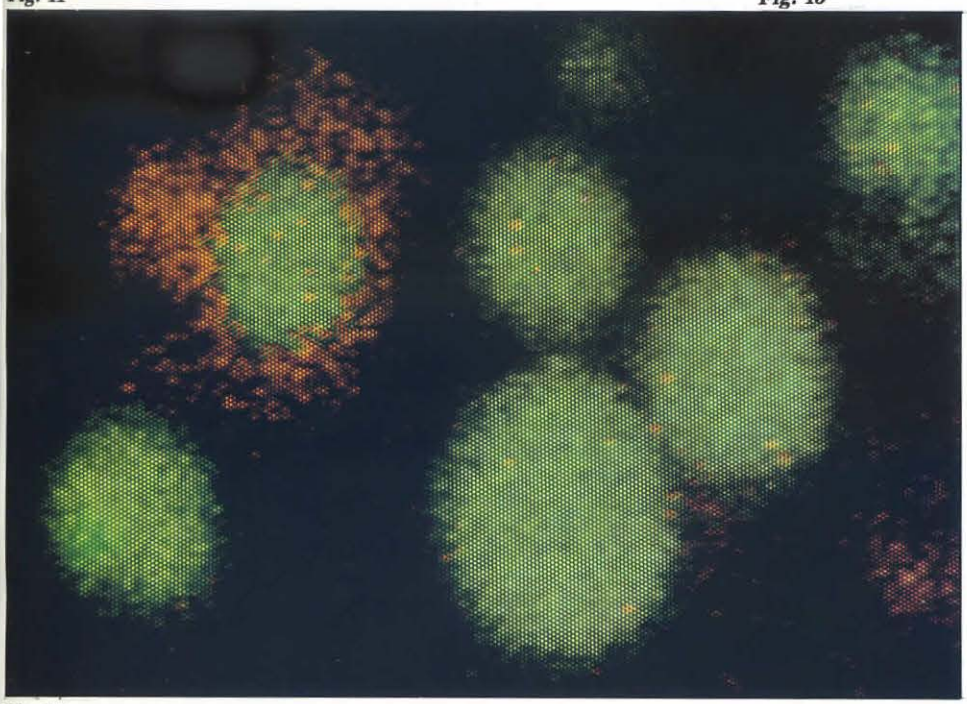

Fig. 12

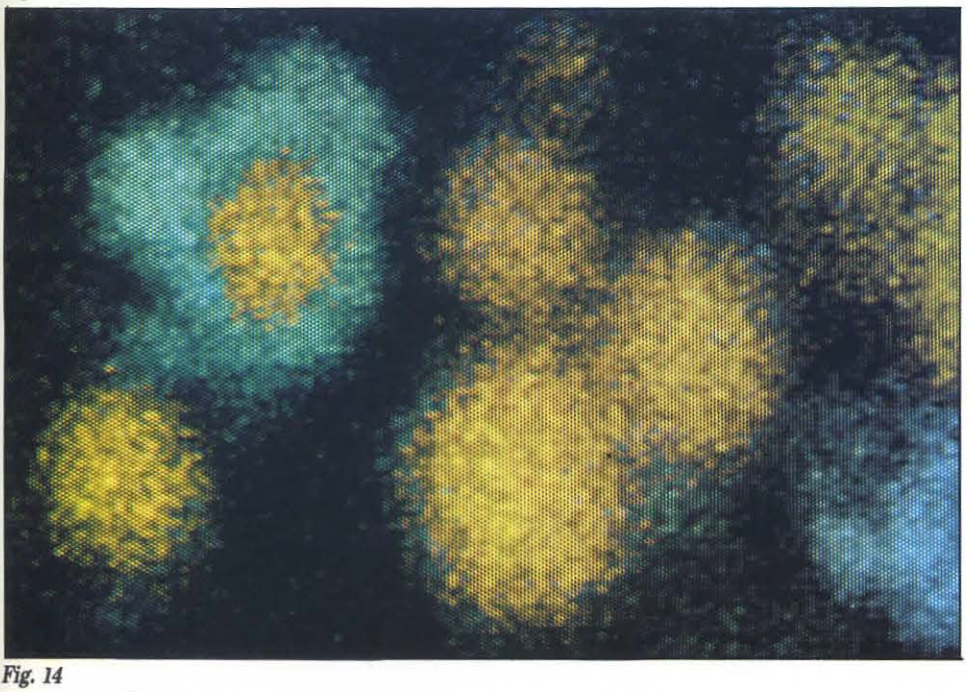

Fig. 14

Figure 11. X-ray maps for copper (Cu) and zinc (Zn) of the area shown in figure 10.

Figure 12. Enlargement of upper central area of figure 11. Note type B granules with copper only and the type B/A granule comprising a type B core with type A periphery containing zinc.

Figure 13. X-ray maps for phosphorus (P) and sulphur (S) of the area shown in figure 10.

Figure 14. Enlargement of upper central area of figure 13.

cell breaks down. Large numbers of type A and B granules can be detected in the faeces of the spider.

Discussion

The *mapping* of electron-generated x-ray photons of different energies as shown in Figs. 8 to 17 has a number of advantages over straightforward spot analyses, in addition to the visual attractiveness of the data!

First, once an area for analysis has been selected and the beam current, spot size, screen resolution and dwell time have been set, the instrument can be left unattended while other work is carried out. High resolution images with long dwell times may take several hours but these can of course be created overnight when the instrument would otherwise be idle. A very clean vacuum in the specimen chamber is essential for such work to prevent the build-up of contamination deposits.

Second, because different elements can be displayed simultaneously in different colours on the same image, it is possible to confirm that the intracellular distribution determined for spot analyses is consistent over whole cells. For example, mapping of the distribution of metals in the granules of woodlice has shown that zinc and phosphorus are always found on the periphery of type B granules containing copper and sulphur, never the other way around (Figs. 11 to 14). Rare exceptions to such general rules, which may have profound significance in physiological terms, may be missed by conventional spot analyses but would be highlighted by mapping.

Third, mapping is particularly useful for interpreting unstained resin-embedded and cryosections which have such poor contrast. Nuclei, for example, can be identified by the arrangement of phosphorus-rich chromatin even though they may not be recognisable as discrete organelles in the microscope. Furthermore, two sections which appear visually identical may have a different elemental composition. Indeed, in another study we have shown that the distribution of phosphorus in the cytoplasm of the liver cells of rats fed on sub-lethal doses of PCBs (polychlorinted biphenyls) is more heterogeneous than in the cytoplasm of control animals. In Fig. 17, it is clear that iron is not distributed evenly in the digestive cells of *Dysdera* and is present at higher concentrations in the cytoplasm than in the type A granules.

Fourth, the techniques can be made quantitative by

Figure 15. Microvillus border of cells in the woodlouse hepatopancreas. Type A material containing calcium, phosphorus and zinc is present on the membranes. The lumen is towards the base of each image. HFW 8μm.

Figure 16. Bright field STEM image and X-ray maps for phosphorus, zinc and calcium in type A granules in a digestive cell of Dysdera crocata. HFW of each map 8μm.

Figure 17. X-ray map for iron of the same area as figure 16. Note that the level of iron is higher in some parts of the cytoplasm than in the granules.

assigning concentrations determined from absolute standards to intensity levels of the pixels. The results reported in this article are not quantitative but it is possible to obtain semi-quantitative information on the levels of the elements relative to each other. For example, two of the x-ray peaks for lead at 2342 (Mα) and 2442 eV (Mβ) interfere with the only peaks for sulphur at 2307 eV (Kα_1), 2322 eV (Kα_4) and 2465 eV (Kβ) so the presence of absence of sulphur from specimens containing lead is difficult to confirm. Nevertheless it is clear that the intensity of the signal for sulphur/lead measured at 2.3 keV in Fig. 8 is stronger in the type B granules on the right of the intercellular membranes than in the type C granules on the left. The intensity of the signal for lead measured at 10.5 keV in Fig. 9 is similar from the two granule types, demonstrating that the level of sulphur in the type B granules (Fig. 8) must be greater than in the type C material.

Conclusions

X-ray microanalytical mapping has enabled us to identify specific pathways for the accumulation of metals in terrestrial invertebrates. Thus, we are now in a position of being able to *predict* the route that metal pollutants will take in a wide range of terrestrial organisms based on the chemical properties of the elements and knowledge of the structure and function of the digestive organs since it is these tissues which first come into contact with contaminants in the diet.

All the types of metal-containing granule described in this article are extremely impermeable; in the case of type A and B granules the embedding resin does not usually penetrate their structure, causing many of them to be torn from thin sections during ultramicrotomy. Moreover, x-ray microanalysis of freeze-dried sections of frozen-hydrated hepatopancreas of woodlice and spiders has shown that granules prepared in this manner have a composition identical to that of resin-embedded granules (with the exceptional of spectra of type A granules in *Dysdera* which contain an additional peak for potassium; the potassium is presumably leached out during conventional embedding procedures). However, metals in the cytoplasm are likely to be much more mobile and further X-ray analyses are required on frozen material before the heterogeneous distribution of specific elements (e.g. Fig. 17) can be confirmed to be an *in vivo* phenomenon.

Our studies have shown that in woodlice, metals such as cadmium and copper are stored permanently by the animals in the S cells (which do not break down) but that iron is lost continuously by the breakdown of the B cells. The rate of excretion of zinc and lead depends on the relative distribution of these metals between the two cell types. The spider *Dysdera*, on the other hand, prevents excessive build up of metals in the hepatopancreas by discharging large numbers of granules from the digestive cells at the end of each digestive cycle. Indeed, calculations show that if *Dysdera* feeding on contaminated woodlice had adopted a permanent storage strategy for zinc and copper, the spiders would be solid brass within a year!

Acknowledgements

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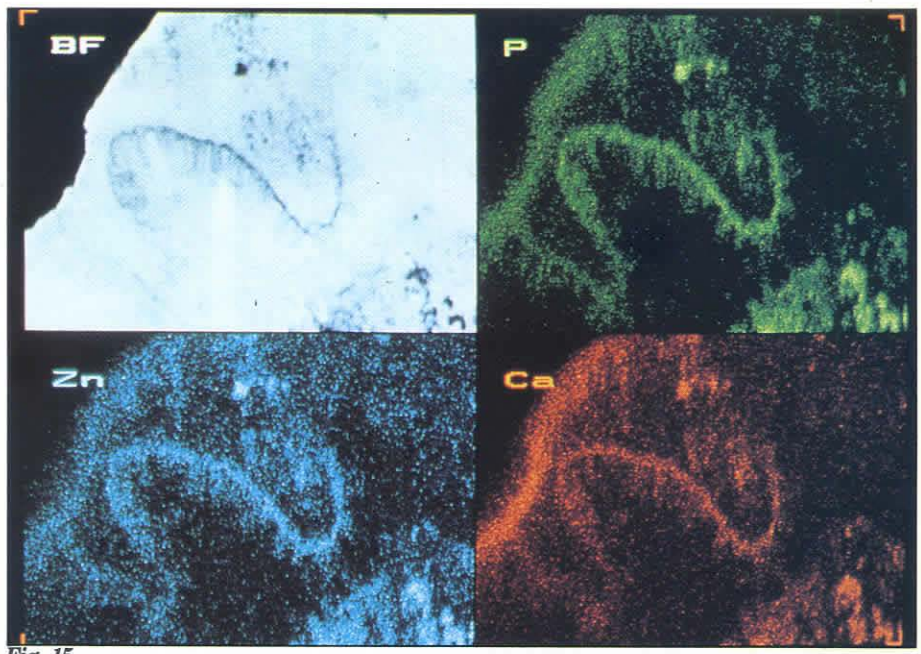

Fig. 15

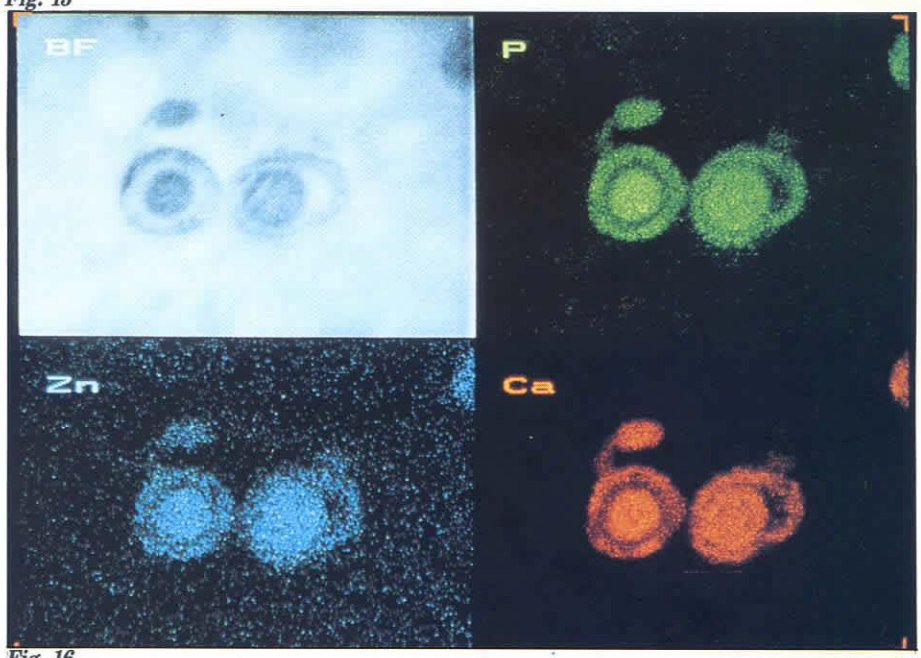

Fig. 16

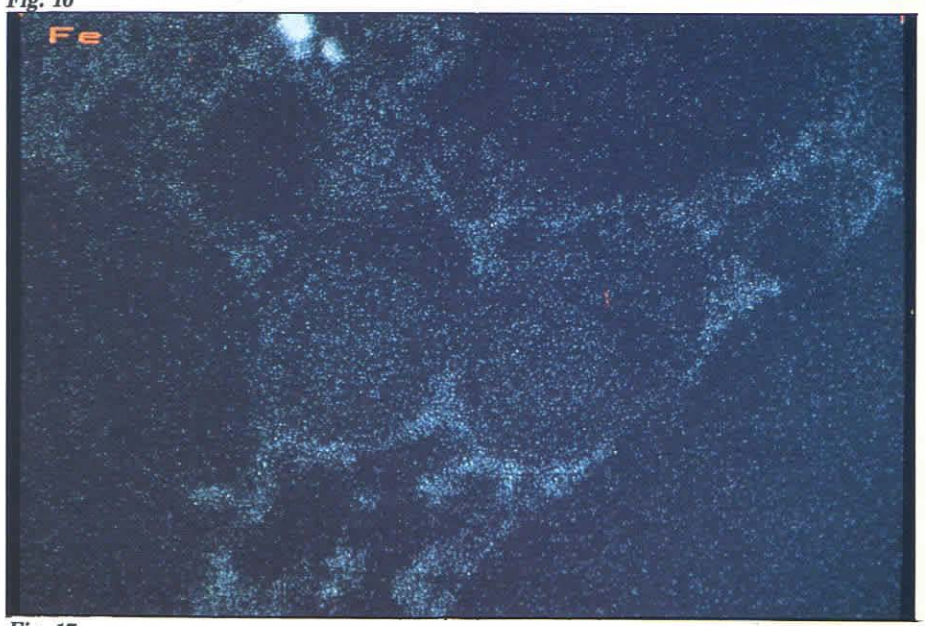

Fig. 17